DNA targeting of rhinal cortex D2 receptor protein reversibly blocks learning of cues that predict reward

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When schedules of several operant trials must be successfully completed to obtain a reward, monkeys quickly learn to adjust their behavioral performance by using visual cues that signal how many trials have been completed and how many remain in the current schedule. Bilateral rhinal (perirhinal and entorhinal) cortex ablations irreversibly prevent this learning. Here, we apply a recombinant DNA technique to investigate the role of dopamine D2 receptor in rhinal cortex for this type of learning. Rhinal cortex was injected with a DNA construct that significantly decreased D2 receptor ligand binding and temporarily produced the same profound learning deficit seen after ablation. However, unlike after ablation, the D2 receptor-targeted, DNA-treated monkeys recovered cue-related learning after 11–19 weeks. Injecting a DNA construct that decreased *N*-methyl-D-aspartate but not D2 receptor ligand binding did not interfere with learning associations between the cues and the schedules. A second D2 receptor-targeted DNA treatment administered after either recovery from a first D2 receptor-targeted DNA treatment (one monkey), after *N*-methyl-D-aspartate receptor-targeted DNA treatment (two monkeys), or after a vector control treatment (one monkey) also induced a learning deficit of similar duration. These results suggest that the D2 receptor in primate rhinal cortex is essential for learning to relate the visual cues to the schedules. The specificity of the receptor manipulation reported here suggests that this approach could be generalized in this or other brain pathways to relate molecular mechanisms to cognitive functions.

perirhinal cortex | entorhinal cortex | antisense | dopamine | *N*-methyl-D-aspartate receptor

Monkeys, as do humans, quickly learn to use visual cues to adjust their behavior based on how much work has been completed and how much remains (the relative workload) before reaching a goal or obtaining a reward (1–4). Because of its strong inputs from the ventral visual pathway and projections to the hippocampal formation (5–13), the rhinal (perirhinal and entorhinal) cortex has been heavily investigated for its role in visual recognition memory (14) and acquisition of stimulus–stimulus associations (15–18). In addition, we became interested in its role in reward-related learning because of its dense innervation by dopamine-rich fibers (19–22), which presumably arise in the substantia nigra pars compacta/ventral tegmental area complex (23). Using a behavioral task, visually cued reward schedules, in which the monkeys are required to perform multiple operant trials to obtain a reward at the end of a schedule, we previously demonstrated that bilateral rhinal cortex ablations prevent monkeys from learning to use visual cues to make the behavioral adjustments in the schedule task (2) and that responses of single neurons in monkey perirhinal cortex reflect a visual cue's relation to the progress through a schedule, i.e., relative workload (3). These latter two studies led us to conclude that monkey rhinal cortex has a critical role in establishing the associations between visual cues and this form of reward contingency.

To test our hypothesis that dopamine, here the D2 receptor, is critical for establishing these associations, we needed a method to manipulate dopamine receptors during the period in which monkeys normally learn to associate visual cues with the relative workload. Until now, pharmacological intervention has been the main approach for connecting receptor mechanisms to behavior. For the experiments we describe here the conventional pharmacological approach using a receptor ligand, in this case an antagonist, is difficult to apply. Pharmacological effects are often relatively short-lived, with half-times of action on the order of minutes to a few hours, whereas the learning process we are studying requires a week or longer to observe (2). In addition, when anatomical localization is required, as desired for our experiments, the pharmacological agent needs to be injected locally and the effect of the agent is generally limited to regions within a few millimeters of the injection sites. In this study we needed to treat the whole rhinal cortex, which is a strip of cortex ≈15 mm long and 7–12 mm wide, depending on the rostral-caudal position. Covering this region adequately with a pharmacological agent would require many injections (≈40; see *Supporting Text*, which is published as supporting information on the PNAS web site), and for agents having a short-lived effect the injections would have to be repeated daily over several weeks of behavioral testing. Although it is likely that each set of injections would cause only a small amount of tissue damage, the cumulative tissue damage over the course of several weeks might become substantial, thereby making inferences about the cause of any behavioral impairment more uncertain.

Given these experimental requirements, we adapted a molecular approach that has been successfully used to decrease ligand binding by the murine D2 receptor (24–26). In the mouse, bilateral intrastriatal injections of a DNA antisense expression construct targeting the D2 receptor were followed by changes in D2-mediated behaviors, including catalepsy and climbing, lasting several weeks. D2 receptor ligand binding was altered in parallel with the behavioral results (25, 26). The specificity and long duration of effects observed when using this technique suggested it might be a particularly attractive approach to apply to learning experiments in primates.

In this study, monkey rhinal cortex was injected with DNA antisense expression constructs designed to interfere with the formation of functional dopamine D2 receptors and/or the *N*-methyl-D-aspartate (NMDA) receptors (24). We then tested whether this treatment impaired learning to associate visual cues with the relative workload. There are two reasons we examined

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Abbreviations: NMDA, *N*-methyl-D-aspartate; nCi, nanocurie.

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dopamine. First, dopamine is thought to be important for reward-related behavior (27–29), and, second, dopamine is present in relatively large amounts in the primate rhinal cortex (19–22). Although the D1 receptor has often been implicated in promoting associative learning, we initially focused on the D2 receptor for two reasons. First, the D2 receptor shows a distinctive distribution with increased density in the deep layers of rhinal cortex, suggesting that this receptor subtype might have a special role in this tissue, whereas the D1 receptor had a more diffuse, and, at peak, less dense, distribution (20, 21). Second, and very important for us, was that the D2 receptor-targeted DNA treatment had a pronounced enough effect on the D2 receptors in a murine model to be followed by large effects on motor behavior (24–26), leading us to believe that the D2-targeting material might be effective in the monkey also. Since our study was undertaken, new information has come to light also showing that the D2 receptor may play an important role in regulating associative learning (30).

The NMDA receptor was chosen as an alternative target because it is also abundant in the rhinal cortex (31), and an alternative, strong hypothesis suggests that NMDA receptors are critical for some aspects of associative learning (32–34). Finally, using two agents provided a means to assess the specificity of the DNA treatments, in the event of a behavioral effect.

In this study injections of DNA constructs into rhinal cortex decreased the amount of ligand binding to the targeted, i.e., D2 or NMDA, receptors. Furthermore, the D2-targeting DNA treatment induced the same behavioral learning deficit as occurs after bilateral rhinal cortex ablation, with the striking difference that the monkeys recover completely several weeks after treatment (11–19). In contrast, NMDA receptor-targeting DNA treatment did not affect the learning. The most parsimonious explanation of our results is that a dopamine-mediated mechanism involving the D2 receptor is essential for learning to associate visual cues with the relative workload.

Materials and Methods

Experimental Apparatus. Rhesus monkeys squatted in a primate chair facing a rear projection screen (90° x 90°) located 57 cm away. A black and white random dot background covered the whole screen. A touch lever, mounted inside the primate chair at waist level, was available to register the monkeys' responses. A spout was placed in front of the monkeys for delivering a drop of water (0.4 ml) as a reward.

Animal Preparation. Seven monkeys were used. Before any training or behavioral testing, each monkey was given a unilateral rhinal cortex removal. This was done to make the injection series somewhat less technically demanding than if both hemispheres needed treatment with DNA. As anticipated based on previous reports in which unilateral cortical ablations were given in monkeys before any training or testing, the monkeys with unilateral rhinal cortex ablations learned our task at the same rate as intact monkeys (see *Supporting Text*). All animal experiments were carried out in accordance with National Institutes of Health guidelines and were approved by the National Institute of Mental Health Animal Care and Use Committee.

Behavior: Visually Cued Reward Schedules. The monkeys were required to perform randomly mixed schedules of one, two, or three identical operant trials to obtain a reward (refs. 1 and 2; Fig. 1*a*). For each trial, when the monkey touched a lever, a visual cue appeared indicating where the current trial was in the current schedule. To complete the trial correctly, the monkey was required to release the lever when a red spot that appeared in the center of the cue changed to green. A blue spot briefly replaced the green spot in correct trials. If the lever was released too early or after the green light disappeared, the trial was counted as incorrect and repeated. A drop of liquid reward was delivered after correct completion of the last trial of a schedule (Fig. 1*a*; see *Supporting Text* for details). The sets of cues are shown in Fig. 1*b*.

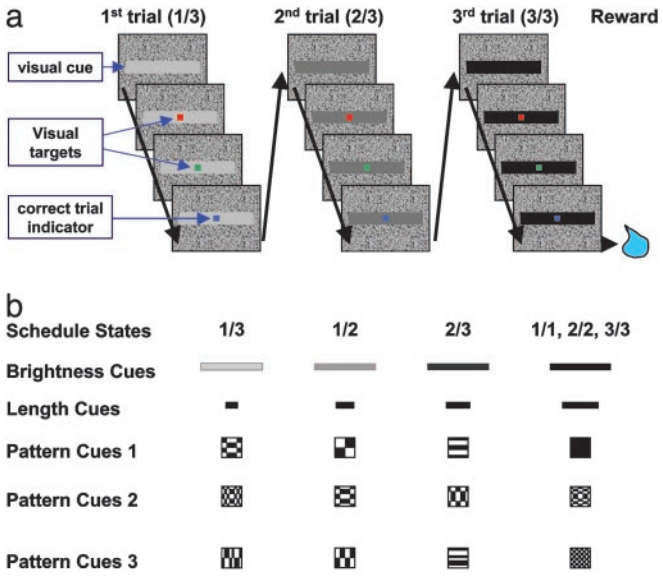

Fig. 1. The visually cued reward schedules task. (*a*) Schematic diagram of a three-trial schedule with brightness cues. On each trial, the monkey was required to respond when a visual target changed from red to green (see *Materials and Methods* for details). The monkey initiated each trial by touching a lever. A trial was scored as correct if the monkey released the lever 200–1,000 ms after the green target (visual target) appeared. If the trial was correctly performed a blue spot (correct trial indicator) replaced the green target. A drop of water (reward) was delivered only after the third trial in the schedule had been correctly completed. A visual cue was assigned to each trial; the cue indicated how many trials had been performed and how many trials remained to be completed before a reward was delivered (relative workload). In the example shown here, the light gray rectangle is the cue for schedule state 1/3, the dark gray rectangle is the cue for state 2/3, and the black rectangle is the cue for state 3/3, where the schedule fraction has the trial number in the numerator and schedule length in the denominator. (*b*) The five visual cue sets used in this study. The schedule states corresponding to the cues are shown in the top row.

Rhinal Cortical Injections. Dopamine D2- and NMDA receptor-targeting DNAs were constructed and injected to cover the rhinal cortex as described in *Supporting Information.*

Receptor Binding Autoradiography. Two experimentally naïve monkeys, each of which received DNA injections of a single type, were used for D2 and NMDA receptor radioligand autoradiography by using standard methods (see *Supporting Text*).

Data Analysis. Behavioral data were collected and analyzed from all sessions in which the monkeys were tested on visually cued reward schedules. Data from each week were combined for analysis. Performance of each individual monkey was evaluated by using the χ^2 test on the numbers of correct and incorrect trials. Group analysis was tested by using repeated measures ANOVA with percent of errors (error rate) from each monkey in each group. For the receptor binding studies, differences in optical density were evaluated by using a paired t test (one-tailed). All statistics were evaluated at $P = 0.05$ levels.

Results

By the second week after introduction of the reward schedules, the number of errors scored by each monkey was directly related

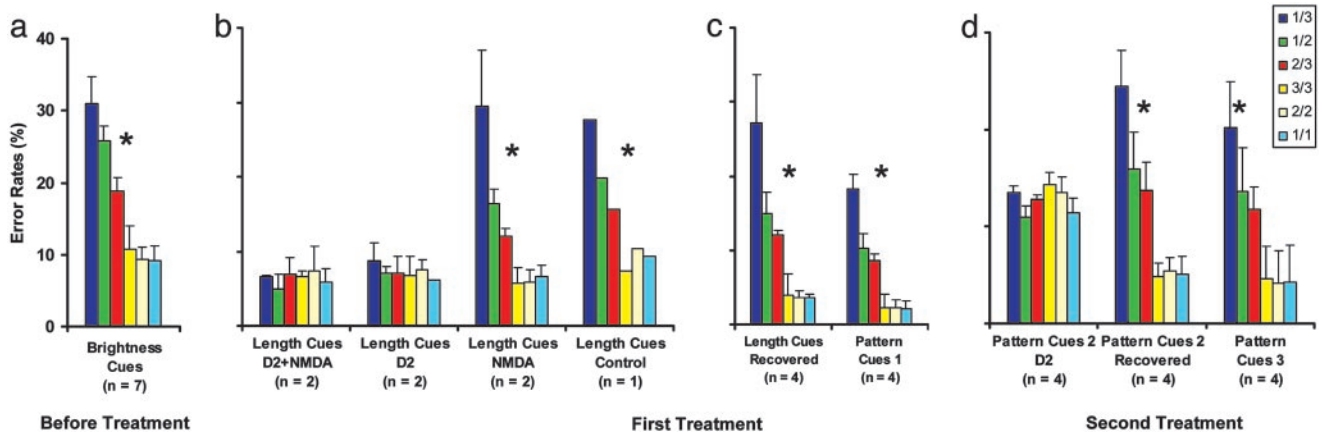

Fig. 2. Error rates of monkeys performing the visually cued reward schedules in the different conditions. Each bar represents the mean error rate for that schedule state; the error bars are SEMs. * marks the conditions in which the error rates were distinguishable (single-factor ANOVA, $P < 0.05$) across the schedule states, indicating that the monkeys were using the cues to adjust their behavior (see text for details). (*a*) Error rates of monkeys using brightness cues in the fourth week of testing after the cue's introduction before any treatment. (*b*) Error rates of different groups of monkeys using length cues in the eighth week after delivery of DNA constructs into the rhinal cortex, i.e., the fourth week after introduction of the length cue. Groups of monkeys were injected with the following: (*i*) a mixture of D2- and NMDA receptor-targeting constructs (Length Cues, D2+NMDA); (*ii*) D2 receptor-targeting construct (Length Cues, D2), (*iii*) NMDA receptor-targeting construct (Length Cues, NMDA), and (*iv*) vector (Length Cues, Control). Monkeys that received either the D2+NMDA receptor-targeting constructs or the D2 receptor-targeting construct were only impaired in learning associations between visual cues and the workload expected before reward. (*c*) Error rates of the four monkeys that received the D2 receptor-targeting construct after behavioral recovery. Data were obtained during the first week after performance had recovered from the effect of either the D2- and NMDA-targeting construct mixture or D2 receptor-targeting construct alone (≈12–20 weeks after injection) (Length Cues, Recovered), and during the third week after new cues have been introduced to the same monkeys (Pattern Cues). (*d*) Error rates of the monkeys that received the second treatment of D2 receptor-targeting construct (D2, $n = 3$; and mixture of D2 and NMDA, $n = 1$). Data (Pattern Cues 2, D2) were obtained during the eighth week after treatment and show that the monkeys did not use the cues to adjust their behavior. Data (Pattern Cues 2, Recovered) collected from the same monkeys during the 12th week after injection, which is the first week after performance had recovered from the effect of the treatments, and during the third week after new cues have been introduced (Pattern Cues 3).

to the number of trials remaining before reward delivery (Fig. 2*a*; for individual data, see Fig. 4, which is published as supporting information on the PNAS web site). The monkeys made progressively fewer errors as the number of trials remaining before reward became smaller (i.e., on trials closer to reward), with the fewest errors occurring in the final, rewarded trial of each schedule. For each of the seven monkeys, the error scores were significantly different across the three nonrewarded schedule states, i.e., 1/3, 1/2, and 2/3 states (χ^2 test, $P < 0.05$ for each monkey; the schedule state fractions, 1/3, 2/3, 3/3, 1/2, 2/2, and 1/1, label trial number in the numerator and schedule length in the denominator). The error scores were statistically indistinguishable in all rewarded schedule states (1/1, 2/2, 3/3), no matter which schedule (one, two, or three trials) was in effect (χ^2 test, $P > 0.05$). Finally, the error score for each of the schedule states was significantly different among the trials within a schedule (χ^2 test, $P < 0.05$). For the entire group of monkeys, the relation between the averaged error rates and schedule states remained the same from the second to the fourth week of testing (interaction term of a two-way ANOVA, $F_{10,125} = 0.329$, $P = 0.97$; Fig. 2*a*). The patterns of learning and performance of the seven monkeys, all of which had unilateral rhinal cortex removals before training began, were similar to those observed in intact monkeys (1, 2), e.g., the learning and performance were indistinguishable from the initial learning scores of the five intact monkeys in our earlier ablation study (ref. 2; interaction term of a two-way ANOVA, $F_{5,71} = 1.74$, $P = 0.14$).

As seen before (1), the error rates were greater in the 1/2 than in the 2/3 state for all seven monkeys (one-tailed paired t test, df = 6, $t = 1.94$, $P < 0.01$). Thus, the error rates depend not only on the number of trials to be completed before reward delivery, but also on the number of trials already completed in the current schedule, i.e., the relative workload. Overall, the patterns of errors indicate that the monkeys used the visual cues to adjust their behavior based on the relative workloads.

After 4 weeks of testing on the reward schedules, each monkey received a set of injections to introduce one of the following four agents into the rhinal cortex of the intact hemisphere: (*i*) DNAs targeting dopamine D2 and NMDA receptors ($n = 2$ monkeys); (*ii*) DNA targeting only the D2 receptor ($n = 2$); (*iii*) DNA targeting the NMDA receptor ($n = 2$); or (*iv*) vector only ($n = 1$). After the DNA injections, each monkey was tested in the same reward schedules but was presented with a new set of cues (length cues; Fig. 1*b*). By the second week, the performance of the three monkeys receiving either the NMDA receptor-targeted treatment or vector-only treatment was the same as before the treatment (Fig. 2*b*). That is, the relationship between the error rates and schedule states obtained with the new length cues was statistically indistinguishable from the relationship observed before treatment (interaction term between schedule states and week of testing in a two-way ANOVA, $F_{5,35} = 1.65$, $P = 0.18$). The behavior was stable from the second to the fourth week (interaction term of a two-way ANOVA, $F_{10,53} = 0.287$, $P = 0.98$). Thus, monkeys receiving treatment targeting rhinal cortex NMDA receptors or only vector learned new cue sets at a rate similar to that measured before the treatment, indicating that these treatments were without effect.

In contrast, all four monkeys receiving a D2 receptor-targeted treatment (combined DNA targeting D2 and NMDA receptors, $n = 2$; DNA targeting the D2 receptor alone, $n = 2$) failed to adjust their error rates across different schedule states for 11–19 weeks after the injections (see *Supporting Text*). The data collected in the fourth week after the introduction of the length cue (eighth week after injection) are shown in Fig. 2*b* (single-factor ANOVA, $F_{5,23} = 0.368$, $P = 0.86$). During the 11–19 weeks after the injections, monkeys receiving DNA constructs targeting the D2 receptor showed the same deficit in associating visual cues with reward schedules as observed in monkeys with bilateral rhinal cortex removals (2). After regaining the ability to use the cues, the behavior was stable; the relationships between the

average error rates and schedule states were the same from the first to the third week after cues were learned (interaction term between schedule states and week of testing in a two-way ANOVA, $F_{10,71} = 0.367$, $P = 0.96$). Thus, although the effect of this DNA treatment lasts for several weeks, it is nonetheless temporary.

To determine whether the relearning was caused by many weeks of practice with a specific cue set, as opposed to recovery from treatment, the four recovered monkeys were presented with another new cue set (pattern cues 1; Fig. 1*b*). After 2 weeks of practice with the new pattern cues, the relationship between error rates and schedule states was indistinguishable from that observed with the initial cue set (brightness cues), before any injections (interaction term of a two-way ANOVA, $F_{5,47} = 0.736$, $P = 0.60$; Fig. 2*c*). This finding, that the ability to learn new cues recovered after treatment and proceeded at the same rate as before DNA treatment, strongly suggests that the D2 receptor-targeted DNA treatment had a time-limited, reversible effect on cognitive behavior.

Finally, as a control procedure intended to determine whether monkeys receiving D2 receptor-targeted treatment could distinguish among the length cues, the rectangles in the length cues were used in place of the red and green spots in the discrimination trials. With the two rectangles having the smallest length difference in the length cues, the monkeys performed the cue discrimination with >90% correct responses in the first testing session.

To demonstrate further that the DNA treatment targeting the D2 receptor was responsible for these reversible behavioral alterations, four of the seven previously injected monkeys (one previously received the D2 targeting construct, two previously received the NMDA targeting construct, and one previously received vector alone; for more detail see Table 1, which is published as supporting information on the PNAS web site) were given a second injection of the combination of D2- and NMDA receptor-targeting construct ($n = 1$) or D2 receptor-targeting constructs alone ($n = 3$). All four of the monkeys showed a prolonged period during which a new set of visual cues (pattern cues 2; Fig. 1*b*) failed to guide their behavior (Fig. 2*d*). As before, all four of these animals learned this cue set after a minimum of 11 weeks after the injections (Table 1). Subsequently, all four monkeys learned a fifth cue set (pattern cues 3; Fig. 1*b*) during the first week after it was introduced.

To test whether our DNA treatments actually affected the targeted receptors, we measured receptor binding by using two experimentally naïve rhesus monkeys (see *Materials and Methods* and *Supporting Text*). Autoradiographs of [^{125}I]iodosulpiride binding in a brain treated with the D2 targeting DNA showed a significant decrease in density of ligand binding in the rhinal cortex of the treated hemisphere compared with the untreated (control) hemisphere (Fig. 3 *a* and *b*; D2 receptor-targeting DNA-treated side, 57.3 ± 3.5 nCi (nCi = nanocuries; mean ± SEM; $n = 36$ measurements per side; control side, 73.1 ± 5.0 nCi; paired *t* test, $t_{47} = -3.5$, $P = 0.0005$).

Autoradiographs of the [^{3}H]MK-801 binding in a brain treated with NMDA receptor-targeted DNA showed a significant decrease in density of ligand binding in the rhinal cortex-treated hemisphere relative to the untreated hemisphere (Fig. 3*c*; NMDA receptor targeting DNA-treated side, 12.8 ± 0.4 nCi; control side, 14.7 ± 0.4 nCi; paired *t* test, $t_{47} = -3.1$, $P = 0.0015$). In addition, the NMDA receptor-targeted DNA treatment did not alter the density of D2 receptor ligand binding (Fig. 3*d*; D2 receptor binding of NMDA receptor-targeted, DNA-treated side, 197.9 ± 10.6 nCi; D2 receptor binding on control side, 200.2 ± 10.2 nCi; paired *t* test, $t_{47} = -0.5$, $P = 0.299$).

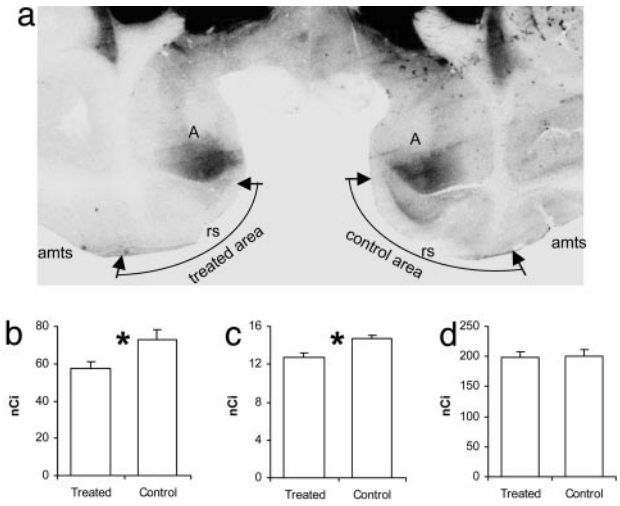

Fig. 3. Receptor binding autoradiography. (*a*) Autoradiograph of a single brain section from the monkey treated with DNA construct targeting the D2 receptor showing D2 receptor ligand binding with [^{125}I]iodosulpiride. The rhinal cortex in the left hemisphere (between the two arrows) was treated with DNA targeting the D2 receptor. There is a significant decrease in D2 receptor ligand binding density in the rhinal cortex of the treated relative to the untreated hemisphere. rs indicates rhinal sulcus, amts indicates anterior middle temporal sulcus, and A marks the amygdala. (*b*) D2 receptor ligand optical density in the rhinal cortex of the monkey treated with the D2-targeting DNA construct. Shown are the means and SEMs for the average density of D2 receptor ligand binding in the D2 receptor construct-treated rhinal cortex (treated) and in the untreated rhinal cortex (control). The treated side has a significantly lower density of D2 receptor ligand binding than the untreated side. (*c*) NMDA receptor ligand optical density in the rhinal cortex of the monkey treated with the NMDA-targeting DNA construct. The NMDA receptor-targeting DNA treatment depresses MK801 binding significantly. (*d*) D2 receptor ligand optical density in the rhinal cortex of the monkey treated with the NMDA receptor-targeting DNA construct. NMDA receptor-targeting DNA treatment did not affect the D2 receptor ligand binding (paired *t* test, $P = 0.6$). This finding indicates that treatment with the DNA construct targeting the NMDA receptor has no nonspecific effect on D2 receptor ligand binding. * indicates a significant difference between two hemispheres (paired *t* test, $P < 0.05$).

Discussion

We have demonstrated that treatment of the rhinal cortex with a DNA construct designed to reduce D2 receptor function leads to a significant, yet reversible, deficit in learning to associate visual cues with the relative workload, i.e., number of trials that have been completed and the number of trials remaining in the current schedule. Strikingly, these animals exhibited recovery of the cognitive function between 11 and 19 weeks after the treatment, whereas in the previous study using bilateral ablations, the monkeys had not recovered 32 weeks after the ablations at which time the study was terminated. Our conclusion that we have produced a reversible learning deficit arising from an alteration of D2 receptor function is supported by two main findings: (*i*) our treatment significantly decreased rhinal cortex D2 receptor ligand binding, and (*ii*) the effect of the treatment was temporary, yet reinstateable.

Behavioral Paradigm. The visual cues provide information about the number of trials to be completed before reward delivery, and in the first trial of a schedule the cue is the only source. The pattern of errors indicates that the monkeys used the visual cues to adjust their behavior. Although we used an operant task and have emphasized a role for rhinal cortex in associating visual cues with workload, it is well recognized that operant tasks often have features in common with Pavlovian tasks, although it is not necessarily straightforward to separate these processes (35).

In the visually cued reward schedules, both the 1/2 and 2/3 conditions are one trial before the rewarded trial, yet intact monkeys make fewer errors in the 2/3 schedule state, showing that the number of trials already completed in a schedule affects the behavioral performance (compare Fig. 3), a conditional relation seen before (1, 2, 4). Thus, the monkey's performance reflects learning the relationship between the visual cues and relative workload, as opposed to time until reward *per se*. How this behavioral pattern depends on the relative interplay between Pavlovian and operant processes remains to be worked out.

Molecular Biological Approach in Monkeys. The expense and limited supply of monkeys makes it impractical to use large numbers of nonhuman primates to conduct extensive cellular and molecular biological characterizations. However, we did obtain ligand histochemistry to evaluate the specificity of the treatments with the constructs on the targeted receptors. The histochemistry shows that the construct treatments significantly reduced the density of specific ligand binding of the targeted receptors. The decrease (≈20% for the D2) in receptor after DNA treatment aimed at the D2 receptor was virtually identical to the decrease (≈18% there) seen after using a similar technique in the striatum of mice that leads to marked changes in motor behavior (25). Thus, although the measured decrease in bound ligand might not seem large, our study is the second circumstance in which this degree of reduction in ligand binding seems to reflect a large enough change in receptor binding to bring about clearly measurable behavioral changes. Furthermore, treatment with the NMDA receptor-targeted DNA lowered the NMDA, but not D2, ligand binding. These points taken together suggest that the decrease in the amount of functional D2 receptor was both large enough to yield an effect and was specific. Although the amount of both D2 and NMDA ligand binding was significantly reduced, our autoradiography results do not permit determination of the absolute number of functional receptors in the targeted tissue because some unbound ligand remained. Nevertheless, it is likely that the behavioral deficit is a consequence of decreased D2 receptor binding. As for the lack of effect of the NMDA material, there are two possible explanations: the NMDA receptors as altered by this material do not play a role in this behavior, or the NMDA manipulation did not lower the receptor density sufficiently to cause an observable behavioral effect in this task.

The behavior of monkeys treated with the DNA targeting the D2 receptor was only temporarily disrupted. There are two reasons that lead us to believe that the behavioral recovery was caused by functional restoration of D2 receptors, rather than by recruitment of other mechanisms or brain regions. First, after each recovery, the monkeys learned subsequent new cue sets at the same rates as intact monkeys. Second, in one of the monkeys a second treatment targeting the D2 receptor in rhinal cortex reinstated the learning deficit. These two findings taken together strongly suggest that the rhinal cortex mediates the new learning of a new cue set after recovery. If so, restoration of D2 receptor function would appear to be the most parsimonious explanation for the recovery of the cognitive abilities assessed here.

It also seems unlikely that the behavioral deficit arose as a consequence of mechanical damage from the injections for three reasons. First, neither the NMDA receptor-targeting DNA treatment nor the vector-alone treatment had an effect, even though the mechanical damage should have been comparable to that after the D2 receptor-targeting treatment. Second, behavioral recovery followed by reinstatement of the deficit and another recovery is inconsistent with permanent effects of mechanical damage (2). Finally, the amount of tissue damage seen on postmortem histological examination of rhinal cortex in our cases used for histochemistry was slight, consisting of needle tracks with slight gliosis around those tracks.

We also considered the possibility that a perceptual deficit interfered with the monkeys' abilities to learn about the cues. Three pieces of evidence argue against this idea. First, the monkeys never displayed any difficulties in the red–green discriminations. In fact, after treatment with the D2 receptor-targeting DNA their performances actually improved, in that they made fewer errors. Second, monkeys that were impaired in learning the associations between cues and workload performed well when the same cues replaced the color targets in the within-trial discrimination. Thus, the monkeys had no difficulty discriminating the visual cues from one another. Third, previous studies have shown that monkeys with bilateral rhinal cortex removals can learn visual discrimination problems at the same rate as intact controls (36–39). Therefore, it is likely that the deficit we observed is specific to learning the associations between visual cues and workload, as opposed to a visual perceptual impairment.

Although our experiments do not reveal how the D2 receptor enables this learning, several recent studies using rodents or rodent tissue slices have shown that the D2 receptor interferes with depotentiation (40), might mediate long-term depression (LTD) (41), might have a role in memory consolidation (42), and might have a role in inducing a short-lived (lasting under 4 h) or "weak" form of long-term potentiation (LTP) (40). The findings in these other studies showing that the D2 receptor has a role in LTP/LTD and memory formation in behavioral experiments makes it of particular interest to design and carry out appropriate physiological experiments to investigate the connection between the D2 receptor and the type of associative learning in our study. Finally, a recent study shows that manipulation of the D2 receptor affects activity of single neurons in prefrontal cortex during a delayed saccade working memory task (43). Wang *et al.* (43) suggest that the activity affected is related to the motor activity related to a saccade rather than being reward related. Given our results showing that the D2 receptor is required to learn that cues are related to reward prediction, it would be more consistent if this prefrontal neural activity was related to having learned the association among the cue, the saccade, and the reward.

Molecular Targeting of Receptors. The precise molecular mechanism(s) of action of DNA antisense constructs is still unidentified, even in the mouse. Regardless, each of our eight DNA treatments targeting the D2 receptor (D2 receptor alone: four monkeys with a total of five treatments; D2+NMDA receptors: three monkeys with one treatment each) was followed by a severe, yet temporary, impairment in learning associations between visual cues and workload. In contrast, DNA treatment targeting the NMDA receptor (two monkeys with one treatment each) or consisting of vector only (one monkey with one treatment) had no effect on learning. This repeated effect of our treatments with the D2-targeting construct suggests a consistent mechanism.

The DNA constructs used in this study were designed to specifically interfere with production of functional D2 or NMDA receptors. One possible mechanism of action is that the receptor-targeted antisense DNA sequence in each construct produced nucleic acid that is complementary to and hybridizes with the respective sequence of cellular D2 or NMDA receptor transcripts. Alternatively, the construct DNA could directly bind to cellular receptor transcripts. Either mechanism could render the targeted transcripts unavailable for translation into normal D2 or NMDA receptor protein (25, 26) through a variety of mechanisms including blocking translation initiation or through the activation of endogenous RNaseH-mediated cleavage of target RNA (44). Our consistent results in macaque monkeys should provide an additional incentive to further characterize the mechanisms by which the specificity of the DNA targeting arises. Approaches using short interfering RNA (siRNA) are also

useful for altering gene expression, but it is not clear what the relationship of the molecular mechanisms of the technique in this study may be to those pathways postulated to be responsible for the effects of siRNA.

In conventional pharmacological studies, the effects of ligands occur because they interact and bind with a receptor. Any lack of specificity for a receptor and the associated side effects occur because the tertiary/quartenary structure of the ligand allows it to bind with different receptor subtypes. In our experiments, the specificity presumably arises because we induce decreases in the amount of a particular receptor protein, thereby reducing the amount of functional receptor. Because it is likely that the mechanism of action is specifically related to some aspect of the nucleic acid sequence, we might expect a relaxed specificity only if the sequences encoding proteins were similar. The NMDA and D2 sequences in our constructs are not homologous (see *Supporting Text*); also, there is no significant homology between the family of D1 and D2 receptor sequences (45), so it seems unlikely that the D2 receptor-targeted DNA affected functioning of the D1 receptor family (including D5). Whether some other dopamine receptor with a sequence similar to the D2 receptor protein might play a role, e.g., the D3 or D4 receptor, remains to be studied.

Because the application of this molecular approach has not to our knowledge been used before in monkeys, it would be valuable to compare the results from this recombinant DNA targeted treatment with the results of instilling classical pharmacological agents targeting the same receptors. The agents we used for the ligand binding experiments, sulpride and MK801, are examples of candidates. However, for reasons provided in the Introduction, it is unlikely that such a series of experiments could be successfully performed. Because of the size and configuration of the rhinal cortex, placement of several injection cannulae would almost certainly be required to treat the entire rhinal cortex, again leading to concern about inducing substantial amounts of mechanical tissue damage.

We have shown that direct injection of a DNA construct interfering with the function of the D2 receptor in the rhinal cortex temporarily leads to a complete inability to learn associations between visual cues and the workload remaining before reward. Thus, it appears that dopamine D2-mediated mechanisms underlie the functional role that monkey rhinal cortex plays in learning this type of association. Future studies can determine whether other types of cognitive behavior dependent on the rhinal cortex likewise depend on D2-mediated mechanisms and also clarify the precise molecular mechanism(s) by which DNA constructs interfere with behavior and receptor ligand binding. Our findings offer a strong incentive for pursuing this recombinant DNA approach as a means to interrogate and modulate the roles of specific components of the molecular pathways underlying behavior.

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1. Bowman, E. M., Aigner, T. G. & Richmond, B. J. (1996) *J. Neurophysiol.* **75,** 1061–1073.
2. Liu, Z., Murray, E. A. & Richmond, B. J. (2000) *Nat. Neurosci.* **3,** 1307–1315.
3. Liu, Z. & Richmond, B. J. (2000) *J. Neurophysiol.* **83,** 1677–1692.
4. Shidara, M. & Richmond, B. J. (2002) *Science* **296,** 1623–1624.
5. Saleem, K. S. & Tanaka, K. (1996) *J. Neurosci.* **16,** 4757–4775.
6. Suzuki, W. A. & Amaral, D. G. (1994) *J. Comp. Neurol.* **350,** 497–533.
7. Baizer, J. S., Desimone, R. & Ungerleider, L. G. (1993) *Visual Neurosci.* **10,** 59–72.
8. Saint-Cyr, J. A., Ungerleider, L. G. & Desimone, R. (1990) *J. Comp. Neurol.* **298,** 129–156.
9. Van Hoesen, G. W., Yeterian, E. H. & Lavizzo-Mourey, R. (1981) *J. Comp. Neurol.* **199,** 205–219.
10. Witter, M. P. & Groenewegen, H. J. (1986) *J. Comp. Neurol.* **252,** 51–77.
11. Aggleton, D. G., Burton, M. J. & Passingham, R. E. (1980) *Brain Res.* **190,** 347–368.
12. Stefanacci, L., Suzuki, W. A. & Amaral, D. G. (1996) *J. Comp. Neurol.* **375,** 552–582.
13. Van Hoesen, G. W. (1981) in *The Amygdaloid Complex: The Differential Distribution, Diversity, and Sprouting of Cortical Projections to the Amygdala in the Rhesus Monkey*, ed. Ben-Ari, Y. (Elsevier, Amsterdam), pp. 77–90.
14. Meunier, M., Bachevalier, J., Mishkin, M. & Murray, E. A. (1993) *J. Neurosci.* **13,** 5418–5432.
15. Murray, E. A., Gaffan, D. & Mishkin, M. (1993) *J. Neurosci.* **13,** 4549–4561.
16. Buckley, M. J. & Gaffan, D. (1998) *Neuropsychologia* **36,** 535–546.
17. Sakai, K. & Miyashita, Y. (1991) *Nature*, **354,** 152–155.
18. Erickson, C. A. & Desimone, R. (1999) *J. Neurosci.* **19,** 10404–10416.
19. Akil, M. & Lewis, D. A. (1993) *Cereb. Cortex* **3,** 533–550.
20. Goldsmith, S. K. & Joyce J. N. (1996) *Neuroscience* **74,** 435–451.
21. Richfield, E. K., Young, A. B. & Penney, J. B. (1989) *J. Comp Neurol.* **286,** 409–426.
22. Berger, B., Trottier, B. S., Verney, C., Gaspar, P. & Alvarez, C. (1988) *J. Comp Neurol.* **273,** 99–119.
23. Insausti, R., Amaral, D. G. & Cowan, W. M. (1987) *J. Comp. Neurol.* **264,** 396–408.
24. Davidkova, G., Zhou, L. W., Morabito, M., Zhang, S. P. & Weiss, B. (1998) *J. Pharmacol. Exp. Ther.* **285,** 1187–1196.
25. Weiss, B., Davidkova, G., Zhou, L. W., Zhang, S. P. & Morabito, M. (1997) *Neurochem. Int.* **31,** 571–580.
26. Weiss, B., Davidkova, G. & Zhou, L. W. (1999) *Cell Mol. Life Sci.* **55,** 334–358.
27. Schultz, W. (2001) *Neuroscientist* **7,** 293–302.
28. Spanagel, R. & Weiss, F. (1999) *Trends Neurosci.* **22,** 521–527.
29. Wise, R. A. (1996) *Curr. Opin. Neurobiol.* **6,** 243–251.
30. Kruzich, P. J. & Grandy, D. K. (2004) *BMC Neurosci.* **5,** 12, www.biomedcentral.com/147-2205/5/12.
31. Kohama, S. G. & Urbanski, H. F. (1997) *Brain Res.* **769,** 44–56.
32. Bear, M. F. & Malenka, R. C. (1994) *Curr. Opin. Neurobiol.* **4,** 389–399.
33. Morris, R. G. M. & Frey, U. (1997) *Philos. Trans. R. Soc. London B* **352,** 1489–1503.
34. Nicoll, R. & Malenka, R. C. (1999) *Ann. N.Y. Acad. Sci.* **868,** 515–525.
35. Shettleworth, S. J. (1998) *Cognition, Evolution, and Behavior* (Oxford, New York).
36. Buckley, M. J. & Gaffan, D. (1997) *Behav. Neurosci.* **111,** 467–475.
37. Eacott, M. J., Gaffan, D. & Murray, E. A. (1994) *Eur. J. Neurosci.* **6,** 1466–1478.
38. Gaffan, D. & Murray, E. A. (1992) *Behav. Neurosci.* **106,** 30–38.
39. Thornton, J. A., Rothblat, L. A. & Murray, E. A. (1997) *J. Neurosci.* **17,** 8536–8549.
40. Manahan-Vaughan, D. & Kulla, A. (2003) *Cereb. Cortex* **13,** 123–135.
41. Chen, Z., Ito, K.-I., Fujii, S., Miura, M., Furuse, H., Sasaki, H., Kaneko, K. & Miyakawa, H. (1996) *Recept. Channels* **4,** 1–8.
42. Setlow, B. & McGaugh J. L. (2000) *Learn Mem.* **7,** 187–191.
43. Wang, M., Vijayraghavan, S. & Goldman-Rakic, P. S. (2004) *Science* **303,** 853–856.
44. Tan, X.-X., Rose, K., Margolin, W. & Chen, Y. (2004) *Biochemistry* **43,** 1111–1117.
45. Missale, C., Nash, S. R., Robinson, S. W., Jaber, M. & Caron, M. G. (1998) *Physiol. Rev.* **78,** 189–225.